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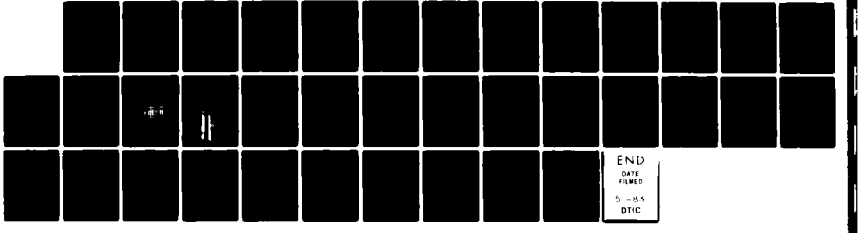

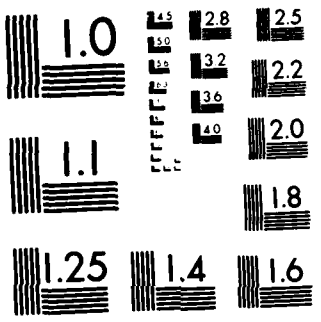

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Report Number 2

Replication of Japanese Encephalitis Virus

Annual Summary Report

By
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Department of Microbiology

January 1981

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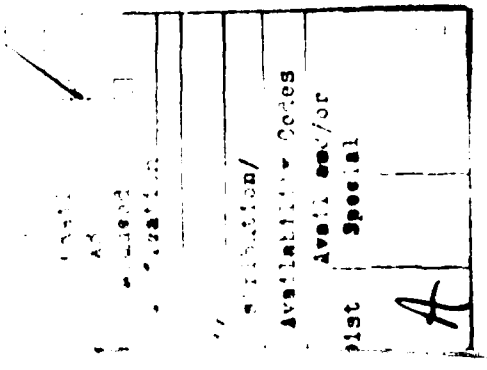
ABSTRACT

Preliminary studies of the structure of Japanese encephalitis virion RNA indicated that the 5'-terminus had a 7-methyl-guanine cap and that the 3'-terminus had little or no polyadenylic acid. Definitive structural studies have not been completed due to difficulties in obtaining sufficient material. Two-dimensional electrophoretic maps of RNase T1-resistant oligonucleotides derived from virion RNA propagated in vertebrate and invertebrate cells were identical. Attempts were made to determine the 3'-terminal base sequence of virion RNA by end-labeling followed by base-specific chemical cleavage. The RNA accepted label poorly by comparison to RNAs from other sources, suggesting the presence of a unique 3'-terminal structure.

Intracellular virus-specific RNA consisted of single-stranded species sedimenting at 40S, 28S, and 15S, and a partially double-stranded fraction which sedimented heterogeneously between 20-26S. It was proposed that the 40S is identical to the genome in function and the 15S and possibly the 28S species are subgenomic mRNAs. Since the smaller RNAs predominate during the eclipse (early) phase of viral growth, it is assumed that they code for non-structural proteins. The double-stranded cores of the 20-26S material sedimented at 18S, 13S, and 9S. Their kinetics of synthesis and accumulation will be studied to determine if they are replicative intermediates or accumulate as end products of RNA synthesis.



Radioimmune precipitation of polypeptides synthesized in infected cells 24-25 hr after infection selected 6-7 virus-specific products ranging in molecular weight from ~10,000 to ~90,000. This method plus polyacrylamide gel electrophoresis of products were demonstrated to be of potential use in identification of virus-coded products in cell-free translation.

Three groups of temperature-sensitive mutants of JEV were obtained by growth of virus in the presence of nucleoside base analogs or by prolonged growth of virus in persistently infected cells. Biochemical and genetic characteristics of the mutants indicated that each group can be utilized in examining particular aspects of virus replication or virus-host interaction.

Mammalian cell cultures persistently infected with JEV were challenged by superinfection at varying multiplicities of infection with homologous standard virus. In general, high multiplicities were sometimes capable of overwhelming the intrinsic interference of the cultures, bringing about cytopathology and death, whereas low multiplicities stimulated production and release of interfering factor(s) thought to be defective virus particles. Although immunofluorescence demonstrated that most cells synthesized viral antigen after superinfection, electron microscopic examination showed that <1% of cells were engaged in viral morphogenetic events, suggesting interference at the level of synthesis or assembly of viral structural components.

Proposals are made to utilize these well-characterized systems in studying the molecular biology of JEV replication.

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Background and Summary

Flaviviruses have a long, unhappy history of producing serious diseases in man. Yet despite the realization that an understanding of the replication mechanisms of these viruses will greatly aid our ability to prevent or treat their diseases (Schlesinger, 1980) and despite recent rapid advances in molecular biological and biochemical methodology, many aspects of flavivirus replication, morphogenesis, and interaction with the host remain unknown, incompletely understood or controversial. Our studies of the replication of Japanese encephalitis virus (JEV) in the past few years have aimed to define the mechanism of multiplication of a prototype flavivirus and to provide possible keys to the control of a serious and widespread disease.

We have approached the problem through the following avenues: (1) structure, synthesis, and function of virus-specific RNAs, (2) intracellular sites of virus replication and morphogenesis, (3) induction and characterization of conditional lethal virus mutants, and (4) persistent infection of mammalian cell cultures by JEV. Our results and hypotheses from previous studies may be briefly summarized as follows.

JEV contains a single-stranded (SS) RNA genome which sediments at 40S. Isolated virion RNA is infectious and is therefore assumed to be a mRNA. It appears to have a 5'-cap, but to lack 3'-poly(A). Intracellular virus-specific RNA consists of the 40S genome, a 20-25S partially double-stranded (DS) replicative intermediate, and SS RNAs which sediment at 28S, 15S, and 12S and are putative subgenomic mRNAs.

Replication occurs wholly in the cytoplasm - the cell nucleus is required only to maintain cellular vitality throughout the

long virus growth cycle. Visible morphogenetic events begin with proliferation of 70-150 mm diameter smooth membrane spheres at 12-15 hr PI. Ultrastructural and chemical evidence suggests that these structures are formed from smooth endoplasmic reticulum and probably condense or coalesce around the material which will become the virus nucleocapsid. Virions mature within cisternae of the endoplasmic reticulum and emerge from cells via exocytosis. No preformed nucleocapsids or budding are seen.

Temperature sensitive (ts) virus mutants have been obtained by mutagenesis and by prolonged growth of virus in persistently infected cultures. They exhibit a variety of biochemically differentiable phenotypes and will be useful in defining steps in replication and virus-host cell interactions.

Two different continuous mammalian cell lines were persistently infected by serial undiluted passage of JEV. The following observations suggest that persistence of this normally cytolytic virus is mediated by production of defective interfering particles. Persistently infected cultures produce small and variable amounts of viral antigen and infectious virus. Neither virus production or released virus is temperature sensitive. Homologous superinfection at low MOI does not alter the cultures; heterologous superinfection results in cell death. No interferon is detectable in cell medium. Concentrated medium from cultures interferes with standard virus replication, yet no truncated RNAs are detectable in persistently infected cells or virions released by them.

These results and the biological systems and methods presently available have led to formation of an outline idea of how JEV replicates. Results from the past year's work have expanded this picture as

detailed in the following report and have suggested further experiments outlined in the proposal. The past year has largely been occupied with development and standardization of procedures and construction and biological analysis of our systems. With these in place, it is proposed to emphasize molecular and biochemical studies in the coming year.

I. Structure, synthesis, and function of virus-specific RNA

A. Structure of virion RNA

Methods were previously described for growth in Vero cells, radioactive labeling, and purification of JEV, from which RNA was extracted and analyzed. Preliminary work indicates that characteristics of the genome include a 5'-methylated cap, probably with type I structure, insignificant polyadenylation, and little or no internal methylation. These conclusions confirm work by other investigators (Dubin et al, 1979; Wengler et al, 1978), and due to difficulties in preparing large amounts of RNA and to concentration of resources on other aspects of the program, work has not progressed beyond this stage.

Oligonucleotide maps of ^{32}P-labeled virion RNA were produced by two-dimensional electrophoretic separation of RNase T1-resistant oligonucleotides in polyacrylamide gels (Dewachter and Fiers, 1972). Virus grown in Vero (African green monkey kidney) and the C6/36 clone of Aedes albopictus cells gave identical patterns (C. Schmaljohn, personal communication). Successful further use of this technique will provide one means of determining structural relationships between standard and defective virus RNAs and the intracellular RNAs from acutely and persistently infected cultures.

Attempts were made to resolve the 3'-terminal base sequence of virion RNA by the method of Peattie (1979) in which the 3'-terminus is labeled by addition of ^{32}P-cytidine in the presence of T4 ligase, then each of the four nucleotides is modified in a separate reaction, and aniline-catalyzed hydrolysis at the modified base produces a nested set of oligonucleotides which can be separated by electrophoresis. However, the 3'-end of purified 40S RNA would not accept the label, although cellular rRNA and Uukuniemi virus mRNA were readily labeled and sequenced under the same conditions (M. Parker, personal communication). This appears to indicate the presence of an unusual structure or configuration at the 3'-end of virion RNA which requires a different approach to sequencing, as outlined in the proposal.

In summary, we are somewhat more familiar with the structure of the JEV genome than a year ago, but will have to apply new approaches, as detailed in the accompanying proposal, to advance our knowledge. One of the problems encountered with each method is the small amount of virion RNA which can be obtained. Possible solutions to this problem are (1) growth and labeling of virus in host cells which produce a higher yield, (2) modification of purification procedures to preserve virion integrity (Obijeski et al, 1976), or (3) reverse transcription of the genome to complementary DNA (Myers, et al, 1977) and amplification by molecular cloning. These are discussed in Methods.

B. Intracellular RNA

Velocity sedimentation in sucrose gradients of intracellular JEV-specific RNA revealed four size classes centered at 40S, 28S,

22S, and 15S. The 22S species was most heterogeneous and up to 50% RNase-resistant, indicating that it is a partially double-stranded (DS) replicative intermediate. The other species were degraded by RNase.

Agarose gel electrophoresis of native intracellular RNA confirmed the presence of a partially DS form which remained near the top of the gel, and SS forms which migrated to positions equivalent to 40S, 15S, and 12S. A species which occurred in variable amounts and was more prominent in some persistently infected cells migrated with or slightly ahead of cellular 28S rRNA and was probably virus-specific. Denaturation by glyoxal in DMSO did not change the relative positions or mobilities of SS-RNAs.

Studies of structural modifications by previously detailed methods indicated that both the 40S and 12-15S species have 5'-methylated caps, and all intracellular RNA species bound to oligo(dT)-cellulose to the extent of about 30%. Both 40S and 12-15S RNAs were found in the infected cell polysomes. We have therefore suggested that the 40S RNA is a mRNA identical to the viral genome and that the 12-15S, and possible the 28S, RNAs are subgenomic mRNAs (Blair and Schmaljohn, manuscript in revision).

It was noted that the predominant virus-specific RNA during the period of maximum synthesis and virus release (18-30 hr PI) was the 40S species. Synthesis of the 15S RNA began 2-3 hr PI and was prominent during the eclipse period (up to 12 hr). The ratio of 15S:40S RNAs varied from 1.3 at 3-9 hr PI to 0.25 at 18-24 hr PI, unlike the alphaviruses, in which the ratio of 26S:40S RNAs remained

constant for a given strain throughout the growth cycle (Kennedy, 1980). Comparison of virus-specific RNA synthesis in different types of cultured cells revealed that 15S RNA was more prominent in Vero and MA-111 cells than in PK-15 or chick embryo cells.

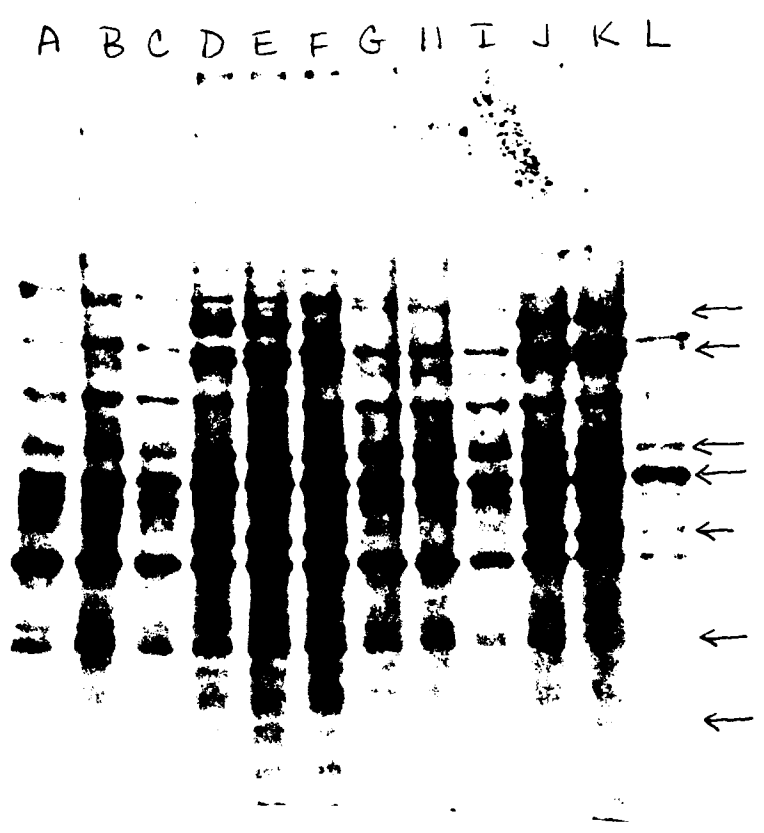
An examination of the replicative intermediate was undertaken to determine the mode of synthesis of the various RNA species. Virus-specific RNA labeled with ^{3}H-uridine from 4-8 hr or 20-24 hr PI was phenol extracted and either directly treated with RNase A or precipitated with 4M LiCl, after which soluble RNA was treated with RNase to degrade the SS portion. The totally RNase-resistant (DS) residual fraction represented only 5-10% of the total labeled material. Velocity sedimentation in 15-30% sucrose gradients revealed that the early DS-RNA contained a heterogeneous 17-24S fraction and a larger amount of 9S material. Late DS-RNA had three symmetrical peaks at 18S, 13S, and 9S. The larger (18S) component may represent the full genome-length replicative form and the smaller species may be involved in synthesis of subgenomic RNAs. Future experiments will determine products of and possible interrelationships between the three forms.

C. Function of RNAs

We have postulated that in addition to the genome, the intracellular subgenomic RNAs which predominate during the eclipse period of JEV are mRNAs. It is probable that these early RNAs are translated into nonstructural proteins which serve in RNA synthesis or control of replication. It follows that the 40S genome serves as a mRNA during its period of maximum synthesis, the exponential growth phase, and codes for structural proteins. This function of

the genome RNA was apparently confirmed by Wengler et al. (1979). In order to determine if these assumptions are valid, it will be necessary to translate each putative mRNA in a cell-free system and to identify its products. We have explored optimum conditions for labeling and characterization by electrophoresis of virus-specific intracellular proteins, and for minimizing the background of cellular proteins, since flaviviruses do not significantly inhibit cell protein synthesis. We observed the effects of administration and removal of hypertonic NaCl (Nuss et al, 1975) followed by a pulse of ^{35}S-methionine (35S-met), and in some experiments superimposed the presence of actinomycin D (act D). Results are shown in Figure 1 of treatment of JEV-infected Vero cells with 0.11 M NaCl (isotonic), 0.22 M, and 0.33 M NaCl for one hr, then pulsing with ^{35}S-met from 24 to 25 hr PI in the presence of 5 μg/ml act D or its absence. It was concluded that 0.22 M NaCl was optimal for preferential reinitiation of virus-specific protein synthesis and that actinomycin did not enhance this effect. Six polypeptides which appeared only in infected cells and with estimated mol wts similar to those given by Shapiro et al (1971) are marked. Their structural or nonstructural designation will be made by coelectrophoresis with virion proteins.

Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of proteins from infected and uninfected Vero cells by the method of Laemmli (1970). Lanes A-F were from cells treated with act D, Lanes G-L were from non-act D-treated cells. Lanes A-C and G-I are from uninfected cells; Lanes D-F and J-L are from JEV-infected. Lanes A, D, G, J received no high salt pulse; Lanes B, E, H, and K received 0.22 M NaCl; Lanes C, F, I, and L received 0.33 M NaCl. Arrows mark virus-specific proteins.

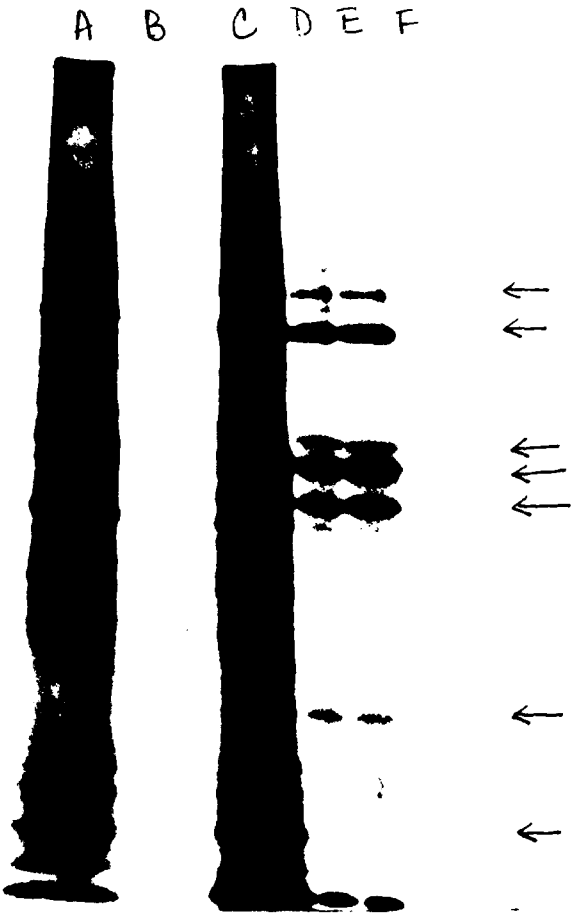


Other results (not shown) indicated that addition of 20 μg/ml cycloheximide to cell culture medium during the salt block enhances the differential between viral and cellular proteins.

Further suppression of the cellular protein background was achieved by immune precipitation of radiolabeled viral proteins

from an infected cell lysate with mouse hyperimmune ascitic fluid (Brugge and Erikson, 1977). In Figure 2, it can be seen that whereas 6-7 polypeptides (of similar size to those marked in Figure 1) were precipitated from infected cell lysates (lanes D & E), no precipitate occurred from uninfected cells (lane B). Similarly, nonimmune mouse ascitic fluid precipitated no polypeptides from infected cells (lane F).

Fig. 2. PAGE of radioimmune precipitates from infected and uninfected Vero cells. Lane A contains untreated material from uninfected cells (identical to Lane B in Fig. 1). Lane B is uninfected cell lysate precipitated with hyperimmune antiJEV mouse ascitic fluid. Lane C is untreated infected cell lysate (identical to Lane E in Fig. 1) Lanes D and E are infected lysate precipitated with antiJEV ascitic fluid. Lane F is infected lysate treated with normal mouse ascitic fluid.



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We are now in a position to translate virus-specific mRNAs in a cell-free system, immunoprecipitate the products, and examine them by electrophoresis, as proposed in Methods. If complete, fully processed polypeptides are produced, they can be readily identified by their mobility in coelectrophoresis with infected cell immune precipitates. If only partial products or unprocessed precursors are produced, their viral specificity will be indicated by immune precipitation and their relationship to known viral proteins will be found by peptide mapping (O'Hare and Nice, 1979).

II. Isolation and characterization of temperature sensitive (ts) mutants of JEV

Isolation of conditional lethal mutants and consequent understanding of viral genetics has several potential values. (1) Identification of the number of essential viral gene products and the role of each in replication. (2) Determination of host functions required for replication. (3) Ascertainment of viral genetic functions required for virulence or cytopathogenicity. (4) Identification of targets for chemotherapy. (5) Production of attenuated virus as a vaccine candidate or gene products for a subunit vaccine.

Much of our effort during the past year has been directed toward isolation and characterization of ts mutants of JEV. We have a large library of mutants from three different sources, each of which may fulfill some of the above functions. The source and properties of each group of mutants will be described separately.

A. Induced ts-host range (hr) mutants.

A group of five mutants was obtained by growth of plaque-purified JEV in Vero cells at 33°C in the presence of 25-100 μg/ml 5-fluorouracil (5-FU) or 5-azacytidine (5-AC) for 48 hr. Virus was plated on LLC-MK2 cells, and over 600 well-isolated plaques were picked, and virus eluted from these was tested for delay or absence of production of cytopathic effects (CPE) in Vero cells at 40°C with concomitant cell killing at 33°C. This initial screening left 50-60 potential ts mutants, but only 5 were stable and showed an efficiency of plating (EOP = PFU at 40°C/PFU at 33°C) $<10^{-2}$ in LLC-MK2 cells. Further characteristics are given in Table 1.

TABLE 1

Mutant	Mutagen	EOP (40C/33C)[a]	RNA Synthesis (%WT)	Reversion[b]	Leak[c]
ts 15	FU	1×10^{-2}	65	1×10^{3}	2×10^{3}
ts 29F	FU	8×10^{-3}	33	<10	30
ts 29N	AC	$<1 \times 10^{-4}$	78	<10	<10
ts 32	AC	$<5 \times 10^{-4}$	118	<10	<10
ts 35	AC	$<4 \times 10^{-3}$	50	<10	<10
WT	-	0.3 - 0.9	100	3×10^{4}	1×10^{3}

[a]Plaque assay in LLC-MK2 cells. When assayed in PK-15 cells, all mutants have EOP similar to WT.

[b]Titer of mutant grown at 40 C for 25 hr and assayed at 40 C.

[c]Titer of mutant grown at 40 C for 24 hr and assayed at 33 C

When the above mutants were assayed in PK-15 cells, a continuous cell line particularly noted for heat resistance, their EOP was equivalent to WT, that is, they did not demonstrate increased temperature sensitivity. At least two other reports of host-

dependent ts mutants have appeared, one for influenza virus (Israel, 1980) and one for vesicular stomatitis virus (Pringle, 1978). In both cases, many of the viral ts lesions were in the polymerase, and their ability to be expressed normally in certain hosts under restrictive conditions was probably due to presence in those permissive cells of host factor(s) which interacted favorably with the viral RNA polymerase. If such is the case with some of the above mutants, a valuable tool for study of host factors required for RNA replication is available.

B. Mutants released from persistently infected cultures

Cloned cultures of MA-111 persistently infected with JEV which were nonproducers of infectious virus were superinfected with either wild type (WT) JEV or ts 32 (see Table 1) and maintained at 33°C. The WT-JEV-infected culture released infectious, non-temperature sensitive virus and did not develop CPE. The ts-infected culture released only ts virus, also without CPE. Both cultures were passed at weekly intervals and assayed periodically for virus release. After 12-15 passes, the WT-infected culture also began to release ts virus. About 50 well-isolated plaques were picked at this time from among virus released by both WT-JEV and ts-JEV-infected cultures. All virus eluted from plaques failed to form plaques at 40°C. Virus clones were further evaluated for RNA phenotype and immunofluorescence pattern with results for some representative isolates given in Table 2.

Mutants with the prefix "WT" were released at passages 12-15 by the culture infected with WT-JEV, those designated "ts" were from the same passage level of the ts32-infected culture.

TABLE 2

Mutant	Titer at 33 C	RNA synthesis at 40C/33°C (%WT)	Immunofluorescence[a]	Leak[b]
WT-JEV	7×10^5	100	90/90	5×10^3
WT 6 (1)	3.5×10^4	113		
WT 6 (4)	9×10^4	121	90/90	
WT 6 (6)	5×10^4	64	25(P)/45	5×10^1
WT 6 (10)	2×10^4	242		1.5×10^2
WT 6 (11)	6×10^4	73	20(P)/30	
WT 1 (4)	3×10^4	76	15/15	
WT 1 (5)	9×10^4	54	25(P)/55	1.2×10^2
WT 1 (8)	8×10^4	388	15/15	5×10^1
WT 1 (7x)	7×10^3	179	65/15	1.7×10^2
WT 3 (5)	1.5×10^4	134	50(P)/90	2×10^2
WT 3 (6)	2×10^5	118	0/15	
WT 3 (11)	3×10^4	169	5/15	
ts 3 (1)	1.5×10^4	149	30/50	
ts 3 (3)	4×10^3	119	25/5	
ts 3 (4)	7×10^4	133	35(P)/35	4×10^2
ts 3 (9)	1×10^4	107	5/20	
ts 3 (11)	8×10^3	46	15/30	
ts 3 (15)	6×10^3	149	15(P)/30	
ts 4 (3)	1×10^5	71		1×10^3
ts 4 (7)	3×10^4	128	50(P)/90	

[a]Percent of cells stained in indirect fluorescent antibody test at 40 C/percent cells stained at 33 C. (P) = patchy fluorescence as opposed to usual smooth appearance.

[b]Titer of virus grown for 24 hr at 40 C and assayed at 33 C.

Unlike ts virus mutants released from cells persistently infected with vesicular stomatitis virus, (Youngner et al, 1976) none of these mutants showed evidence of an RNA^- phenotype (inability to synthesize virus-specific RNA at the nonpermissive temperature.) In fact, most appeared to synthesize RNA more efficiently at 40°C than WT virus, and some [WT6(10), WT1(8)] were "super RNA" mutants. Immunofluorescence studies suggested some mutants may have protein synthesis, transport, or assembly defects

(cf. Saraste et al, 1980). PAGE patterns of proteins synthesized at 40°C by some mutants are presently being examined. This group of mutants displayed equal temperature sensitivity in all host cells tested, including LLC-MK2, Vero, MA-111, CHO and PK-15.

The variety of mutant phenotypes, particularly as demonstrated by immunofluorescence, suggested that the above virus isolates bore genetic lesions in different cistrons, and complementation was attempted between a few mutants selected for phenotypic diversity. MA-111 cultures were co-infected with equal MOI (~1) of each of two different mutants, incubated at 40°C for 24 hr, and the yields assayed by plaquing at 33°C. Complementation indexes were calculated by dividing the yield of the mixed infection by the sum of the yields of each single infection. Complementation indexes displayed in Table 3 below suggested interference rather than complementation had occurred. Despite the two-fold plaque purification of each mutant before use, interference may have resulted from presence in virus stocks or generation during growth of defective interfering virus derived from the persistently infected cultures (see Section III).

TABLE 3

	WT 1 (8)	WT 6 (10)	WT 6 (6)	ts 3 (4)	ts 4 (3)	WT 1 (5)
WT 1 (8)	--	0.1	0.2	0.2	0.1	0.1
WT 6 (10)		--	0.3	0.4		0.04
WT 6 (6)			--	0.4	0.02	0.06
ts 3 (4)				--	0.1	0.02
ts 4 3)					--	0.2
WT 1 (5)						--

The conditions under which these mutants arose - growth in persistently infected host cells, reduced growth temperature, etc. - in some respects mimic the production of virus by arthropod vectors. Further study of the chemical and biological properties of these viruses may aid our understanding of the effects of such selective pressures on disease agents.

The absolute temperature sensitivity of these mutants and their failure to exhibit complementation may indicate that they bear multiple mutations. Virulence and immunogenicity testing in laboratory animals will be conducted in order to determine their possible candidature for attenuated vaccine.

C. Mutagen-induced ts mutants

A third library of mutants is currently being tested. These were induced by growth of twice plaque-purified WT-JEV in PK-15 cells in the presence of 100-200 μg/ml 5-FU or 5-AC. All growth and assays of mutants have been conducted in this same cell line.

After 48-96 hr growth in mutagen, virus titer was reduced by 10^{-3} to 10^{-5} from that of parallel untreated cultures. Mutagenized virus was plaqued, well-isolated plaques were picked, and virus eluted from plaques was used to infect cells in duplicate microtiter plates. One plate was incubated at 33°C until CPE developed, the duplicate was incubated at 40°C. Wells which developed CPE at 40°C slowly or not at all were considered to contain potential mutants, and stocks were plaqued to determine EOP. Of 947 plaques originally picked, 51 were potential mutants. Of these, 9 have been established as stable mutants and 19 are still being tested. Characteristics of the 9 mutants are given in Table 4 below.

TABLE 4

Mutant	Mutagen	Titer at 33°C	Titer at 40°C	EOP	Leak	Reversion
68-1	AC	3×10^{6}	$<10^{2}$	$<3.3\times10^{-5}$		
128-1-1	FU	1.3×10^{6}	$<10^{1}$	$<8\times10^{-6}$	5×10^{4}	0
96-1-1	FU	3×10^{6}	6×10^{3}	2×10^{-3}		
92-1-1	FU	4×10^{6}	$<10^{1}$	$<3\times10^{-6}$	5×10^{3}	0
90-1-1	FU	5.5×10^{6}	$<10^{2}$	$<2\times10^{-5}$		
32-1-1	FU	3.5×10^{6}	3.5×10^{4}	10^{-2}		
13-1-2	FU	1×10^{6}	$<10^{1}$	$<1\times10^{-5}$	2×10^{3}	0
96-1-2	FU	2×10^{7}	$<10^{2}$	$<5\times10^{-6}$		
32-1-2	FU	5.8×10^{6}	1.8×10^{2}	3×10^{-4}		
132-1-1	FU	1×10^{6}	$<10^{2}$	$<1\times10^{-4}$		

A pilot complementation test was conducted in the same manner as described above. Resultant complementation indexes, given in Table 4A below, did not suggest interference, but showed no evidence of complementation.

TABLE 4A

	ts 13	ts 92	ts 128
ts 13	-	0.645	0.508
ts 92		-	1.56

III. Persistent infection of cultured mammalian cells by JEV.

Establishment over 5 years ago of JEV persistence in two mammalian cell lines demonstrated the ability of this normally cytolytic virus to establish a commensal relationship with cells of its vertebrate as well as its arthropod hosts, and initiated our exploration of the virus and host factors which contribute to the stability of such systems.

Previous research pointed out the following characteristics of the systems. Rabbit kidney (MA-111) cells became persistently infected after 30-40 serial undiluted passages of virus. African green monkey kidney (Vero) cells were persistently infected by exposure to virus released from the stabilized MA-111 cultures. Both types released low and variable amounts of infectious virus continuously. Examination by immunofluorescence or infectious

center assay demonstrated that 10-90% of cells in cultures produced viral antigen and 1-10% of cells released infectious virus. Interferon was not detectable in medium of persistently infected cultures, and superinfection by heterologous virus resulted in cell death. Homologous superinfection did not produce CPE or cell death. Concentrated medium from persistently infected cells interfered with replication of standard JEV, but no truncated RNA species were found in released virions or in cells (Schmaljohn and Blair, 1977).

To further characterize the extent and nature of virus infection, over 200 cells were cloned from parental persistently infected cultures and evaluated for production of virus antigen and infectious virus. Over 98% of cells were nonproducers of virus or viral protein, yet when superinfected with standard JEV, became chronic virus producers indistinguishable from parental persistently infected cultures. Virus specific RNA was detectable in nonproducer cell clones, but no method was found for successful induction of latent virus replication (Schmaljohn and Blair, 1979).

During the past year, our studies have centered on the nonproducer cell clones and their response to homologous superinfection. To determine if infectious virus release after superinfection involved replication of the latent genome or only of the superinfecting genome, nonproducer clones were superinfected with JEV ts-32 and incubated at 33°C, the permissive temperature for the mutant. All virus released was temperature sensitive, whereas superinfection of the same clones with WT-JEV resulted in release of virus which grew at both 33°C and 40°C. We concluded that superinfection did not induce replication of the persistent genome.

About 10 of the nonproducer clones have been maintained in passage for over a year. Despite our inability to artificially induce virus replication in these cultures, several began spontaneous release of virus at various stages after passage 25. In a few cases, virus production ceased again after a few weeks, but most have continued to release low levels of virus for 30-50 passages. Concomitant with spontaneous initiation of virus release was production in 100% of the cells of viral antigen.

The following series of experiments was directed toward identification and characterization of factors which interfered with replication of latent or superinfecting virus and consequent protection of cells from CPE.

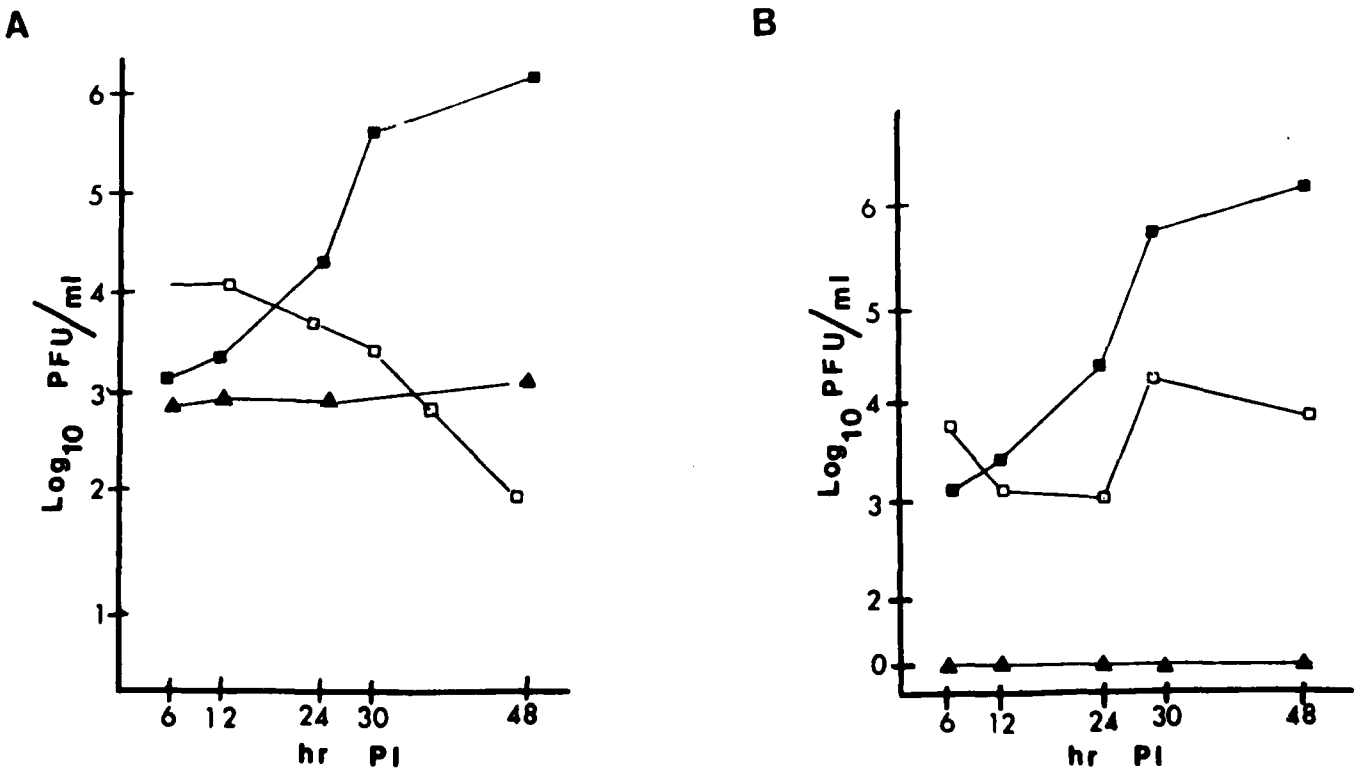
Nonproducer or spontaneous virus producer cell clones were superinfected with various multiplicities of infection (MOI) of WT-JEV. As shown in the examples in Table 5, multiplicities of ≤ 1 invariably resulted in chronic virus release, whereas higher MOI sometimes overwhelmed the inherent interference to cause cell death.

TABLE 5

Cell clone	Development of CPE at given superinfection MOI		
	10	1	0.1
MC15 p52 (nonproducer)	+	-	-
MC167 p 53 (producer)	+	-	-
VCL20 p43 (nonproducer)	-	-	-
VC132 p33 (producer)	+	-	-

Released virus was titrated by plaque assay over a period of time after low MOI superinfection of either nonproducer or producer clones. As shown in the graphs in Figure 3, superinfection resulted in an initial increase in infectious virus release which rapidly declined to a low level chronic production. These observations suggested that superinfection triggered increased production of an interfering factor.

Fig. 3. Growth curves of JEV in acute infection of Vero cells (■), before superinfection of persistently infected cells (▲), and after superinfection of persistently infected cells (□). A. Virus producer persistently infected cell clone. B. Nonproducer persistently infected cell clone.



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To our knowledge, only the work of Darnell and Koprowski (1974) and our previous publications (Schmaljohn and Blair, 1977; 1979) have suggested that flaviviruses may generate interfering particles under certain conditions. In order to further document this phenomenon, we assayed the ability of medium from persistently infected cultures to cause interference.

Ability of factor(s) in the medium of persistently infected clones to interfere with replication of WT-JEV was titrated in the following manner. Persistently infected culture medium was mixed with an equal volume of WT-JEV stock, diluted in a 10-fold series, and plated on uninfected Vero or LLC-MK2 cells. In order to allow maximum interaction and coinfection between defective and standard virus, cultures were incubated in liquid medium and observed daily for development of CPE. Control assays were of diluted culture medium alone (infectivity) and WT-JEV stock alone (control). Table 6 illustrates representative results. Interference with replication of WT-JEV is demonstrable only after superinfection (although autointerference is observed before superinfection). Interference is denoted by a reduction in endpoint titer in mixed infection as compared to the parallel control titration and appears to be associated with a titratable particle released into culture medium.

TABLE 6

Cell clone	Assay Type	CPE Development										
		Before superinfection					After superinfection					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
MC15 p52	Infectivity	-	-	+	-	-	+	+	-	-	-	-
	Interference	+	+	+	+	-	-	+	+	-	-	-
	Control	+	+	+	+	-	+	+	+	+	+	-
MC167 p65	Infectivity	-	+	-	-	-	-	+	-	-	-	-
	Interference	+	+	+	+	-	-	+	+	-	-	-
	Control	+	+	+	+	-	+	+	+	+	+	-
VC132 p73	Infectivity	+	-	-	-	-	-	-	-	-	-	-
	Interference	+	+	+	-	-	-	+	-	-	-	-
	Control	+	+	+	+	-	+	+	+	-	-	-
VC120 p60	Infectivity	-	-	-	-	-	+	-	-	-	-	-
	Interference	+	+	+	+	-	+	-	-	-	-	-
	Control	+	+	+	+	-	+	+	+	+	-	-

Demonstration of the possible presence of defective interfering (DI) particles in medium of cloned persistently infected cells, as well as observations of spontaneous release of infectious virus and production of viral antigen by all cells leads to the conclusion that every cell in each persistently infected culture is infected, even though most are blocked or restrained in some aspect of virus replication. Nevertheless, an electron microscopic study of nonproducer clone cells revealed no evidence of virus replication in hundreds of cells examined. Even after superinfection, only ~1% of cells so examined contained ultrastructural features associated with virus morphogenesis (Leary and Blair, 1980). These consisted mainly of aberrent proliferation of smooth cytoplasmic membranes and intracytoplasmic accumulation of smooth membrane spheres. Very few mature virions were observed, by contrast to acute infection by JEV (Schmaljohn, Happ, and Blair, submitted). These observations have led to the conclusion that the stage at which interference operates in persistently infected cultures occurs prior to normal virus morphogenesis. Low levels of 40S RNA in persistently infected cells (Blair and Schmaljohm, in revision) suggest that genome synthesis may be blocked. Future studies will characterize the particles which are released into medium and appear to mediate interference.

Our research has resulted in a steadily increasing knowledge of normal Japanese encephalitis virus structure and replication.

The systems developed and analyzed during the past year, summarized above, provide examples in which virus expression is selectively modified or inhibited. By comparative use of these systems in experiments described in our renewal proposal, we plan to further describe the steps in virus replication and interaction with hosts.

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